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The proliferative effect of estrogen on mammary cells has been linked to the production of mitogenic growth factors and increased expression of surface receptor tyrosine kinases. Growth factor binding to receptor tyrosine kinases activates multiple, interactive signaling pathways, most of which involve sequential activation of serine/threonine protein kinases. Retinoic acid (RA) inhibits signaling between receptor tyrosine kinases and the nucleus. At least two signaling pathways stimulated by receptor tyrosine kinase utilize protein kinase C (PKC) family members as mediators. Because of the relationships between hormone-dependent mammary cell proliferation, RA growth regulation and protein kinase C, **we hypothesized that retinoic acid inhibits the uncontrolled proliferation of mammary carcinoma cells by increasing the activity of PKCα.** This hypothesis was tested by studying the effect of RA on expression of PKCα, and by studying the effects of constitutively expressing PKCα (in the absence and presence of RA) on two breast cancer cell lines are used in our study, the hormone-dependent/RA-sensitive T47D cell line and the hormone-independent/RA-resistant MDA-MB-231 line. The results of these studies demonstrated that:

- retinoids induce expression of PKCα
- PKCα activity is required for retinoids to arrest proliferation of hormone dependent, human breast cancer cells
- PKCα inhibits mitogenic signaling from receptor tyrosine kinases to promoters of selected proto-oncogenes
- PKCα can confer retinoid sensitivity on hormone-independent, retinoid-resistant, human breast cancer cells
- retinoids prevent down-regulation of active PKCα

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FOREWORD

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INTRODUCTION

Retinoids have anti-tumor effects and a number of clinical trials of retinoids in human breast cancer have been initiated. Although retinoids inhibit proliferation of numerous breast cancer cell lines, responsiveness appears to be limited to estrogen-dependent cells, in which retinoic acid (RA) inhibits estrogen stimulation of proliferation. The proliferative effect of estrogen on mammary cells has been linked to the production of mitogenic growth factors and increased expression of surface receptor tyrosine kinases. Growth factor binding to receptor tyrosine kinases activates multiple, interactive signaling pathways, most of which involve sequential activation of serine/threonine protein kinases. Retinoic acid (RA) inhibits signaling between receptor tyrosine kinases and the nucleus. The components of these signaling pathways that serve as targets for RA have yet to be identified. At least two signaling pathways stimulated by receptor tyrosine kinase utilize protein kinase C (PKC) family members as mediators. Because of the relationships between hormone-dependent mammary cell proliferation, RA growth regulation and protein kinase C, **we have hypothesized that retinoic acid inhibits the uncontrolled proliferation of mammary carcinoma cells, by increasing the overall activity of PKCα, and/or decreasing the overall activity of PKCζ.** Two HBC cell lines are used in our study, the hormone-dependent/RA-sensitive T47D cell line and the hormone-independent/RA-resistant MDA-MB-231 line.

BODY

Overview. This award, DAMD17-96-1-6022, provided support for post-doctoral training of Dr. Yunhi Cho. The research proposal was divided into two specific aims that were to be completed by performing four tasks. Tasks 1-3 were completed in Years 1 & 2 and the results have been reported in past annual reports. Task 4 was begun in Year 2 and completed in Year 3. One paper describing these results has been published and 2 additional manuscripts will be submitted within the next 4 weeks. In addition Dr. Cho presented her data at a lecture to the Food and Nutrition Research Institute of EWHA Women's University (proceedings published as: Cho, Y. and Talmage, D.A. The role protein kinase C in anti-cancer mechanism of retinoic acid. Foods and Nutrition Proceedings, vol. 8, Department of Foods and Nutrition, Asia Food and Nutrition Research Institute, EWHA Women's University, Seoul Korea. 1998, pp. 63-80), at the 8th Asian Congress of Nutrition in Seoul, Korea (Aug 29 – Sept. 2, 1999) and will present it at an upcoming Keystone Symposium (Protein Kinase C: Structure, Regulation and Cellular Function, 2/5/00 – 2/10/00). At the end of the training period (September 1, 1999) Dr. Cho was promoted to the position of Associate Research Scientist, a research track faculty position at Columbia University. As of March 1, 2000 Dr. Cho will become Assistant Professor of Nutritional Biochemistry at the Graduate School of East-West Medical Science, Kyung Hee University in Korea. On the whole, I think that it is clear that this training fellowship has been enormously successful.

Detailed Report on Scientific Progress. Progress on Task 1: Characterization of cell lines for expression, translocation and activity of PKC isozymes. Two parental cell lines have been compared. One is hormone-dependent and retinoid sensitive (T47D cells), the other is hormone-independent and retinoid resistant (MDA-MB-231 cells). PKC expression patterns were analyzed by a combination of northern blot and immunoblot analyses. These results have been published in Cho et al., J. Cellular Physiology, 1997, 172:306-313.

Table 1. Expression of PKC isozymes in human breast cancer cells.

	T47D				MDA-MB-231			
	-RA		+RA		-RA		+RA	
	RNA	Protein	RNA	Protein	RNA	Protein	RNA	Protein
PKCα	-	-	+	+	+	-	+	-
PKCβ	-	-	-	-	-	-	-	-
PKCγ	-	-	-	-	-	-	-	-
PKCδ	+	+	+	+	+	+	+	+
PKCε	+	+	+	+	+	+	+	+
PKCζ	+	+	-	-	+	+	+	+

Note: Changes in PKCα were seen within 24 hr of RA treatment of T47D cells whereas changes in PKCζ levels were not seen until 5+ days of RA treatment. No significant differences in the expression of PKCδ or PKCε were seen under any conditions.

Based on the above results future experiments focussed on the role of PKCα.

Progress on Task 2: Characterization of cell lines for proto-oncogene expression. We used northern blot analyses to characterize the expression of 6 proto-oncogenes in response to mitogenic stimulus of T47D and MDA-MB-231 cells both in the absence and presence of RA. Estrogen, epidermal growth factor (EGF), insulin and fetal bovine serum rapidly induced expression of c-myc, c-fos, fosB, c-jun and junB but not junD in T47D cells. Prior treatment with RA (>12 hours) reduced induction in each case by 60-80%. EGF was significantly more potent at inducing expression of these genes than other agents tested. Only fetal bovine serum effectively induced expression of these target genes in MDA-MB-231 cells. RA pretreatment had no effect on fbs induction of any gene examined except for junB. RA pretreatment reduced junB induction by 3 fold. These results are described in: Tighe, A.P., Cho, Y. and Talmage, D.A. Retinoic acid inhibits human breast cancer cell proliferation by attenuating epidermal growth factor signaling. Manuscript submitted to Mol. Cell. Biol., Jan., 2000.

Progress on Task 3: Characterization of retinoic acid effect on cell cycle progression. Retinoids inhibit proliferation of T47D cells in a time and dose dependent manner. At 10^{-6} M, RA inhibited proliferation within 24 hr, at 10^{-9} M inhibition was maximal at 72 hr. Retinoids at any concentration tested, had no effect on proliferation of MDA-MB-231 cells. Retinoid inhibition of T47D cell proliferation was the apparent result of a loss of mitogenic signaling resulting in cells reversibly entering a G_0-like phase of the cell cycle. These results are also reported in Tighe, A.P., Cho, Y. and Talmage, D.A. Retinoic acid inhibits human breast cancer cell proliferation by attenuating epidermal growth factor signaling. Manuscript submitted to Mol. Cell. Biol., Jan., 2000.

Progress on Task 4: Manipulation of individual PKC isozyme gene expression. **T47D cells.** PKCα was constitutively expressed in T47D cells. The resultant cell line, αT47D grew poorly (at a rate roughly equivalent to parental cells treated with 10^{-8} M RA). Inhibition of PKCα activity in both αT47D cells and in RA treated T47D cells returned proliferation rates to control levels. Therefore the anti-proliferative effect of retinoids on these cells was mediated by the retinoid induced gene product, PKCα. This result has been published (Cho et al., J. Cellular Physiology, 1997, 172:306-313). The role of PKCα as a mediator of retinoid action is conclusively supported by results of extensive comparative analysis of the effects of retinoid and constitutive PKCα expression on EGF signaling (± a PKCα inhibitor). These latter studies were not carried out by Dr. Cho but are

reported in a manuscript on which she is a co-author (Tighe, A.P., Cho, Y. and Talmage, D.A. Retinoic acid inhibits human breast cancer cell proliferation by attenuating epidermal growth factor signaling. Submitted).

MDA-MB-231 cells. PKCα was constitutively expressed in MDA-MB-231 cells. PKCα expression had no effect on MDA-MB-231 cell proliferation rates. PKCα expression also failed to affect the ability of fetal bovine serum to induce expression of c-fos, c-jun or fosB. PKCα expression marginally reduced serum induction of junB mRNA levels. What PKCα expression in MDA-MB-231 cells did do, was to confer sensitivity to retinoids. Thus, although RA had no effect on MDA-MB-231 proliferation or serum signaling, RA treatment did inhibit the proliferation of PKCα expressing MDA-MB-231 cells and inhibited fetal bovine serum induction of c-fos and junB. RA treatment also increased PKCα levels in these cells. Recent experiments demonstrate that this increase was the result of increasing the half-life of active PKCα. The mechanism for this effect is not known. These results are reported in: Cho, Y. and Talmage, D.A. Protein kinase Cα expression confers retinoic acid sensitivity in MDA-MB-231 human breast cancer cells. Manuscript submitted to Mol. Cell. Biol., Jan., 2000.

APPENDED MATERIAL.

Key accomplishments.

- Demonstrated that retinoids induce expression of PKCα
- Demonstrated that PKCα activity is required for retinoids to arrest proliferation of hormone dependent, human breast cancer cells
- Demonstrated that PKCα inhibits mitogenic signaling from receptor tyrosine kinases to promoters of selected proto-oncogenes
- Demonstrated that PKCα can confer retinoid sensitivity on hormone-independent, retinoid-resistant, human breast cancer cells
- Demonstrated that retinoids can prevent down-regulation of active PKCα

Reportable outcomes.

Manuscripts etc.:

⇒Cho, Y., Tighe, A.P. and Talmage, D.A. Retinoic acid induced growth arrest of human breast carcinoma cells is accompanied by induction of protein kinase Cα expression. J. Cell. Physiol., 1997, 172:306-313.

⇒Cho, Y. and Talmage, D.A. Protein kinase Cα expression confers retinoic acid sensitivity in MDA-MB-231 human breast cancer cells. Manuscript submitted to Mol. Cell. Biol., Jan., 2000.

⇒Tighe, A.P., Cho, Y. and Talmage, D.A. Retinoic acid inhibits human breast cancer cell proliferation by attenuating epidermal growth factor signaling. Manuscript submitted to Mol. Cell. Biol., Jan., 2000.

→ Cho, Y. and Talmage, D.A. The role protein kinase C in anti-cancer mechanism of retinoic acid. Foods and Nutrition Proceedings, Department of Foods and Nutrition, Asia Food and Nutrition Res. Inst. EWHA Women's University, Seoul Korea. 1998, 8:63-80.

→Cho, Y and Talmage, D.A. Protein kinase Cα expression repairs retinoic acid insensitivity of growth arrest in MDA-MB-231 cells. 8th Asian Congress of Nutrition: Good Nutrition for All! – New era for nutrition rights. Seoul, Korea, 8/29/99 – 9/2/99.

→Cho, Y. and Talmage, D.A. Protein kinase Cα expression confers retinoic acid sensitivity in MDA-MB-231 human breast cancer cells. Keystone Symposia on Molecular and Cellular

Biology, "Protein Kinase C: Structure, Regulation and Cellular Function. Taos, NM. 2/5/00 – 2/10/00.

Patents.
None.

Degrees obtained.
None.

Cell lines developed.
αT47D – T47D human breast cancer cells, constitutively expressing PKCα and resistance to G418.
αMDA – MDA-MB-231 human breast cancer cells constitutively expressing PKCα and hygromycin resistance.
OPR-PKCα-MDA – MDA-MB-231 human breast cancer cells constitutively expressing the bacterial lac I gene and both hygromycin and G418 resistance. In addition these cells reversibly express PKCα following addition of IPTG to the culture media.

Informatics.
None.

Funding applied for.
None, yet. NIH R01 application is being prepared.

Employment.
Dr. Cho has accepted a position as an Assistant Professor in the Department of Nutritional Biochemistry at the Graduate School of East-West Medical Science, Kyung Hee University, Korea.